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PURIFICATION AND PROPERTIES OF TUNA SUPERNATANT AND MITOCHONDRIAL MALATE DEHYDROGENASES

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SUMMARY

The supernatant and mitochondrial forms of malate dehydrogenase (L-malate: DPN oxidoreductase, EC 1.1.1.37) have been purified from heart tissues of the Pacific yellow-fin tuna, *Neothunnus macropterus*. The mitochondrial enzyme was crystallized and was found to be homogeneous on ultracentrifugal analysis, having an $s_{20,w}$ of 4.0. The molecular weights of both supernatant and mitochondrial enzymes were estimated to be 67 000 by chromatography on calibrated gel-filtration columns. The supernatant and mitochondrial enzymes could readily be differentiated on the basis of their susceptibility to substrate inhibition by oxaloacetate and by their reactivity with coenzyme analogs. The mitochondrial enzyme was found to be more thermolabile than the supernatant enzyme. A rabbit antiserum directed against the tuna mitochondrial enzyme readily inhibited this enzyme and reacted strongly with it, as judged by double diffusion in agar and by microcomplement fixation. The antimitochondrial malate dehydrogenase serum failed to inhibit or cross-react with the tuna supernatant enzyme, although the serum cross-reacted with the mitochondrial malate dehydrogenases of other fish.

INTRODUCTION

In early studies on the intracellular distribution of malate dehydrogenase (L-malate:DPN oxidoreductase, EC 1.1.1.37) in animal tissues, enzymatic activity was detected in both the mitochondrial and high-speed supernatant fractions[1,2]. Kinetic evidence that the enzymatic activity in these two cellular fractions might reside in distinct proteins was first provided by DELBRÜCK *et al.*[3,4]. Further studies on the malate dehydrogenases from a number of different species have shown that the supernatant and mitochondrial malate dehydrogenases could readily be distinguished as

Abbreviations: APDPN, 3-acetylpyridine analog of DPN; TNDPN, 3-thionicotinamide analog of DPN; DeDPN, nicotinamide hypoxanthine analog of DPN.

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separate entities on the basis of their physicochemical, catalytic, and immunological properties.

The present communication reports the purification of supernatant and mitochondrial malate dehydrogenases from the heart tissues of the Pacific yellow-fin tuna, *Neothunnus macropterus*, and some of the catalytic, physical, and immunological properties of these enzymes.

EXPERIMENTAL

Materials

L-Malic acid and oxaloacetate were purchased from Nutritional Biochemicals Corporation, β-mercaptoethanol from the Eastman Kodak Company, DEAE-cellulose from the Brown Company, and Sephadex G-100 from Pharmacia, Incorporated. Pyridine nucleotide coenzymes and coenzyme analogs were obtained from P-L Biochemicals.

Hydrolyzed starch was from Connaught Laboratories, Toronto; and phenazine methosulfate and nitro blue tetrazolium were purchased from Mann Research Laboratories. Other chemicals were of reagent grade.

Frozen tuna hearts were obtained commercially, and fresh tissue was obtained through the courtesy of the U.S. Bureau of Commercial Fisheries Technological Laboratory, Gloucester, Mass. Crystalline chicken supernatant and mitochondrial malate dehydrogenases and pig mitochondrial malate dehydrogenase were prepared in this laboratory by methods to be described elsewhere.

Enzyme assays

Assays were performed in a Zeiss spectrophotometer PMQ II, at 25°, at the wavelengths of the absorption maxima of the reduced coenzymes: 340 mμ for DPNH and DeDPNH; 365 mμ for APDPNH; 400 mμ for TNDPNH. Routine assays were performed at pH 7.5 in 0.1 M phosphate buffer, containing 0.1 mg/ml of DPNH and $3 \cdot 10^{-4}$ M oxaloacetate. Assays employing DPN^+ or the oxidized coenzyme analogs were performed in 0.1 M sodium pyrophosphate buffer (pH 8.9) containing 0.033 M sodium L-malate and coenzyme concentrations of 0.3 mg/ml. A unit of malate dehydrogenase activity is defined as the amount of enzyme required to oxidize or reduce 1 μmole of coenzyme per min under the conditions described.

The temperature stability of the tuna mitochondrial and supernatant malate dehydrogenases was determined by diluting the enzymes, with the complement fixation buffer described below, to obtain 10 malate dehydrogenase units/ml, and heating these solutions at various temperatures. Temperature was controlled to within $\pm$ 0.1°. Samples were withdrawn at various times, chilled immediately, and assayed for malate dehydrogenase activity. Both crystalline tuna mitochondrial malate dehydrogenase and a sample of mitochondrial malate dehydrogenase purified to the same extent as the supernatant malate dehydrogenase gave identical rates of inactivation.

Determination of protein content

In purification of the enzymes, protein was determined spectrophotometrically by measuring the absorption of light at wavelengths of 280 and 260 mμ, as described

by WARBURG AND CHRISTIAN[5]. For experiments with the crystalline tuna mitochondrial malate dehydrogenase the experimentally determined extinction coefficient was used.

Starch-gel electrophoresis

Gel electrophoresis was performed as described by FINE AND COSTELLO[6] using phosphate–citrate buffer at pH's 7.0 and 8.0 and veronal buffer at pH 8.5. Regions of enzymatic activity were located on the gels using a tetrazolium staining mixture specific for malate dehydrogenase[7]. Protein was stained on duplicate slices of the gels with an amido-schwartz dye[8].

Ultracentrifugal analysis and molecular weight determinations

Ultracentrifugal analysis of the tuna mitochondrial malate dehydrogenase was carried out in a Beckman-Spinco Model E ultracentrifuge equipped with a Philpot–Svensson optical system. The sedimentation constant was determined at a rotor speed of 59 780 rev./min and at a temperature of 21.4°. The value was corrected to water at 20°. The protein concentration was approx. 12 mg/ml in 0.1 M phosphate buffer (pH 7.2).

The molecular weights of the tuna mitochondrial malate dehydrogenase and supernatant malate dehydrogenase were estimated by chromatography on Sephadex G-100 columns calibrated for molecular weight determination, using proteins of known molecular weight, as described by ANDREWS[9]. Full details of the methods employed and the proteins used for calibration will be presented elsewhere.

Tryptic digestion and peptide mapping

Tryptic digestion and peptide mapping of tuna mitochondrial malate dehydrogenase were performed by methods previously employed in this laboratory for lactate dehydrogenases[10]. Ehrlich's stain, specific for tryptophan[11], was used to examine peptide maps for the presence of this amino acid.

Fluorescence studies

The fluorescence spectrum of tuna mitochondrial malate dehydrogenase was determined on a Zeiss spectrofluorometer Model ZFM 4c. Ribonuclease, chicken-heart mitochondrial malate dehydrogenase, chicken-heart lactate dehydrogenase, and tyrosine were used as standards. The spectra were corrected for variations in the photomultiplier efficiency, and monochromater dispersion, with varying wavelengths.

Amino acid composition

The amino acid composition of tuna mitochondrial malate dehydrogenase was determined on a Beckman automatic amino acid analyzer using the procedure of MOORE, SPACKMAN AND STEIN[12]. Samples of an $(NH_4)_2SO_4$ suspension of the tuna mitochondrial malate dehydrogenase were dialyzed overnight against three changes (2 l) of 0.1 M phosphate buffer (pH 7.5) to displace the $(NH_4)_2SO_4$. The enzyme solutions were then dialyzed exhaustively against glass-distilled water to remove the phosphate buffer, prior to lyophilization and hydrolysis. Hydrolysis times of 24 and 48 h with 6.0 M HCl were used. The values of serine and threonine were corrected to zero time of hydrolysis by dividing the 24-h values by 0.90 for serine and 0.95 for threonine[13].

Immunological procedures

Rabbit antisera against crystalline tuna mitochondrial malate dehydrogenase were prepared in the following manner. Ten mg of crystalline enzyme in 1.0 ml of normal saline were mixed with an equal volume of Freund's adjuvant and injected into the toepads and thigh muscles. First course sera were obtained after 2 weeks. The rabbits then received intravenous injections of 10 mg of enzyme (in solution), dispensed as 4 portions of 2.5 mg during an 8-day period. Second course sera were obtained 6 days after the last injection. The rabbits then received intraperitoneal injections of 10 mg of enzyme (in solution) injected over a 4-day period as described above. Third course sera were obtained on the 6th and 7th days after the last injection. Fourth course sera were obtained after further series of intravenous injections as described above.

The antisera were characterized as follows. Double diffusion tests were performed by the OUCHTERLONY method[14] in 1% agar containing 0.9% NaCl and 0.2 mg/ml of powdered methiolate, and adjusted to pH 8.0. The antigen wells contained 0.1 ml of undiluted antiserum. One antigen well contained 0.1 ml of crystalline tuna mitochondrial malate dehydrogenase solution (100 units/ml), and the other, 0.1 ml of a crude tuna-heart tissue extract (diluted to contain 100 units/ml). The wells were about 0.6 cm apart, and diffusion was allowed to continue for at least 2 days at 5°. After this time the agar plates were washed briefly with buffer and placed in a tetrazolium staining mixture specific for malate dehydrogenase[7], to ascertain whether the precipitin band and the band of malate dehydrogenase activity were coincident. The rabbit antisera prepared against tuna mitochondrial malate dehydrogenase gave a single sharp precipitin band both with the crystalline enzyme and with the crude tissue extract. The precipitin band stained for malate dehydrogenase activity.

Microcomplement fixation was performed according to WASSERMAN AND LEVINE[15], using the 7.0-ml total reaction volume described in their Footnote 3. All reagents were diluted in a buffer mixture containing 0.14 M NaCl, 0.01 M Tris (pH 7.5), $5 \cdot 10^{-4}$ M $MgSO_4$, $1.5 \cdot 10^{-4}$ M $CaCl_2$ and 0.1% bovine serum albumin, the final pH being adjusted to 7.5. The rabbit antisera to tuna mitochondrial malate dehydrogenase gave a single peak of fixation over a wide range of antigen concentration.

Inhibition of malate dehydrogenase activity by antisera was measured as described by WILSON AND KAPLAN[16], using the complement fixation buffer described above as diluent.

RESULTS

Enzyme purification

Since lactate dehydrogenase was to be isolated from the same batch of tuna hearts as were to be used for the purification of malate dehydrogenases, the following procedure was designed both to give an efficient extraction of the two types of enzyme and to effect their early separation. Pilot fractionations were carried out on small samples of the enzyme solutions before each subsequent step.

Step 1. Extraction of tissues. 8.3 kg of frozen tuna hearts were thawed and then ground 3 times in a mechanical meat grinder. The minced tissues were then suspended

in 20 l of 0.1 M Tris + 0.001 M EDTA + 0.001 M β-mercaptoethanol (pH 7.6) at 4°, and stirred slowly for 3 h. The mince was filtered through cheesecloth and the filtrate was clarified by centrifugation. Sonication of a sample of the residues from these steps produced no appreciable release of malate dehydrogenase activity, indicating that the majority of the mitochondria had been disrupted by the freeze-thawing and grinding. All subsequent purification was carried out at a temperature of 4°.

Step 2. $(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ was slowly added to the tissue extract from *Step 1* to obtain 50% satn.*. In this, and in all subsequent steps involving $(NH_4)_2SO_4$ fractionation, the pH was kept at 7.5 by the gradual addition of NH_4OH.

After 3 h the suspension was filtered on fluted filter papers, and the precipitate was discarded. More solid $(NH_4)_2SO_4$ was added to the filtrate to give 75% satn., and the suspension was left for several hours and then filtered on fluted filter papers. The moist precipitate was scraped off and dissolved in 750 ml of 0.005 M Tris + 0.001 M EDTA + 0.001 M β-mercaptoethanol (pH 7.6). Little malate dehydrogenase activity was detected in the filtrate.

Step 3. First DEAE-cellulose chromatography. The dissolved precipitate from *Step 2* was dialyzed overnight against 3 changes (20 l each) of 0.005 M Tris + 0.001 M EDTA + 0.001 M β-mercaptoethanol (pH 7.6). The dialyzed enzyme was placed on a 15 cm × 75 cm DEAE-cellulose column, prepared as described by PESCE *et al.*[13] and previously equilibrated with the above buffer. The column was eluted with the same buffer until the eluent showed negligible 280-mμ absorbing material. No malate dehydrogenase or lactate dehydrogenase activity was eluted at this time. The column was then eluted with an exponential NaCl gradient (in 0.005 M Tris + 0.001 M EDTA + 0.001 M β-mercaptoethanol (pH 7.6)), the final salt concentration being 0.2 M. Lactate dehydrogenase activity was eluted early in the gradient, while the malate dehydrogenase was more strongly adsorbed to the resin and was eluted at higher salt concentrations, the two enzymatic activities being completely separated. All fractions showing appreciable malate dehydrogenase activity were combined, and solid $(NH_4)_2SO_4$ was added to 70% satn. After 3 h the suspension was centrifuged at 20 000 × *g* for 30 min and the precipitate was dissolved in 0.005 M Tris + 0.001 M EDTA + 0.001 M β-mercaptoethanol (pH 7.6). There was no appreciable malate dehydrogenase activity in the supernatant fraction, which was discarded. Starch-gel electrophoresis showed the presence of both supernatant and mitochondrial malate dehydrogenase in the precipitate.

Step 4. Second DEAE-cellulose chromatography. The precipitate from *Step 3* was dialyzed overnight against 3 changes of 0.005 M Tris + 0.001 M EDTA + 0.001 M β-mercaptoethanol (pH 7.6), and placed on an 8 cm × 60 cm DEAE-cellulose column, previously equilibrated with the above buffer. Elution was as in *Step 3*, except that the gradient was linear. Two peaks of malate dehydrogenase activity were eluted from the column. The first peak to be eluted was identified as supernatant malate dehydrogenase, and the second as mitochondrial malate dehydrogenase on the basis of their electrophoretic mobility. Fractions containing the supernatant malate dehydrogenase and mitochondrial malate dehydrogenase activities were separately pooled, and these enzymes were precipitated by raising the

* % saturation of $(NH_4)_2SO_4$ was based on Table I of S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, 1955, p. 76, even though the enzyme solutions were kept at 4°.

$(NH_4)_2SO_4$ concentration to 70% satn. The precipitates were dissolved in 0.005 M Tris + 0.001 M EDTA + 0.001 M β-mercaptoethanol (pH 7.6). At this point further attempts to purify aliquots of the supernatant malate dehydrogenase only resulted in drastic losses of of enzymatic activity. The remaining supernatant malate dehydrogenase was therefore retained at this level of purification (approx. 30-fold) and used as such in the experiments described below. It was stored at 4° in 50% $(NH_4)_2SO_4$ containing 0.01 M Tris + 0.001 M EDTA + 0.01 M β-mercaptoethanol (pH 7.5) under which conditions it was quite stable over a period of several weeks.

Step 5. Further purification of tuna mitochondrial malate dehydrogenase. The precipitate of mitochondrial malate dehydrogenase from the previous step was dark brown in color. This coloration was removed by further column chromatography on DEAE-cellulose (8 cm × 60 cm) as described in *Step 4*. A colored component overlapped slightly with the peak of malate dehydrogenase activity and, at the loss of some enzyme, only fractions having minimal discoloration were pooled*. The pooled malate dehydrogenase fractions were concentrated and the enzyme precipitated by dialysis against satd. $(NH_4)_2SO_4$ containing 0.001 M EDTA + 0.001 M β-mercaptoethanol and adjusted to pH 7.5. The precipitate was collected by centrifugation at 20 000 × *g* for 15 min and dissolved in a minimal amount of 0.05 M Tris + 0.001 M EDTA + 0.001 M β-mercaptoethanol (pH 7.5). Solid $(NH_4)_2SO_4$ was added to 50% satn. and the last traces of an amorphous, inactive colored protein removed by centrifugation. Further solid $(NH_4)_2SO_4$ was added slowly over a period of several hours until the enzyme started to crystallize at approx. 60% satn. Crystallization was allowed to proceed for 2 days, after which time the crystals were harvested by centrifugation. Tuna mitochondrial malate dehydrogenase was recrystallized 3 times by the same procedure and stored at 4° as a crystalline suspension in $(NH_4)_2SO_4$ containing 0.001 M EDTA + 0.001 M β-mercaptoethanol, adjusted to pH 7.5.

A yield of approx. 150 mg of crystalline enzyme was obtained. The gain in specific activity over the crude tissue extract was 290-fold.

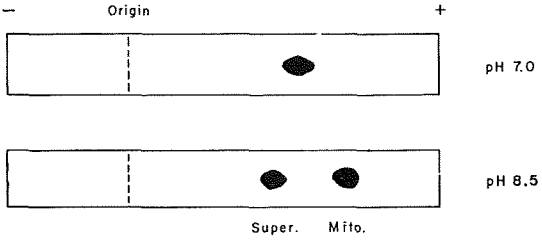
The crystalline tuna mitochondrial malate dehydrogenase was characterized by ultracentrifugal properties, amino acid analysis, peptide mapping and immunological properties. The properties of the crystalline mitochondrial malate dehydrogenase were compared with those of the partially purified supernatant, malate dehydrogenase.

Starch-gel electrophoresis

When crude extracts of tuna heart were examined by starch-gel electrophoresis at pH 7.0 in phosphate–citrate buffer, only one slightly smeared band of enzymatic activity was observed, which migrated toward the anode. This is unlike the situation observed with mammalian and avian tissue extracts, where the mitochondrial and supernatant malate dehydrogenases are readily separable by electrophoresis at this pH (refs. 7, 17). Electrophoresis of the tuna extracts at pH 8.0 in phosphate–citrate buffer, or better, at pH 8.5, in veronal buffer, showed two bands

* Subsequent studies have shown that the colored component can be effectively separated from tuna mitochondrial malate dehydrogenase by chromatography on long (100 cm) columns of Sephadex G-100, which obviates the requirement for the more complicated DEAE-cellulose procedure described.

of malate dehydrogenase activity (Fig. 1). By cellular fractionation studies with fresh tuna hearts, the more anodal band was identified as being mitochondrial in origin, while the more cathodal band was identified as supernatant malate dehydrogenase. This is the reverse of the electrophoretic pattern observed with mammalian and avian malate dehydrogenases, where the supernatant malate dehydrogenase has the most anodal migration. Furthermore, the tuna mitochondrial malate dehydrogenase did not show the multiple electrophoretic forms typical of the mammalian and avian enzyme[7,18,19]. When a concentrated solution of the crystalline tuna



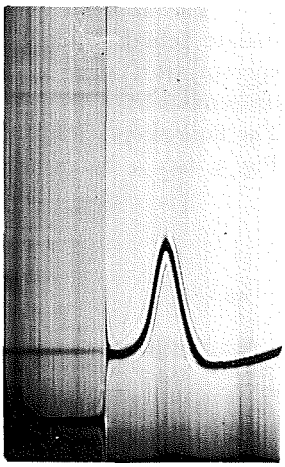


Fig. 1. Tracings of starch-gel electrophoretic patterns of a mixture of tuna supernatant and mitochondrial malate dehydrogenases at pH 7.0 and at pH 8.6.

Fig. 2. Sedimentation velocity pattern of crystalline tuna mitochondrial malate dehydrogenase. The protein concentration was approx. 10 mg/ml in 0.1 M phosphate buffer (pH 7.0). Rotor speed 59 780 rev./min; temp. 25°; bar angle 60°.

mitochondrial malate dehydrogenase was subjected to electrophoresis at pH 8.6 two very minor enzymatically active electrophoretic components were observed which had a slightly faster anodal migration than the major electrophoretic form. These minor components were estimated to account for less than 5% of the total enzymatic activity. A duplicate slice of the starch gel of the crystalline mitochondrial malate dehydrogenase was stained for protein. The only protein bands observed were those which coincided with the bands having malate dehydrogenase activity.

Ultracentrifugal analysis

The crystalline tuna mitochondrial malate dehydrogenase was examined in the ultracentrifuge using schlieren optics. The enzyme sedimented as a single symmetrical peak, as shown in Fig. 2, with an $s_{20,w}$ of $4.0 \cdot 10^{-13}$ cm·sec^{-1}. This sedimentation constant is in excellent agreement with those obtained for pig, horse, and beef mitochondrial malate dehydrogenases[20–22].

Molecular weight determinations

The molecular weights of the tuna supernatant and mitochondrial malate

dehydrogenases were determined by chromatography on Sephadex G-100 gel filtration columns which had been calibrated for molecular weight determination, with proteins of known molecular weight[9]. Both tuna malate dehydrogenases had identical elution volumes, which corresponds to a mol. wt. of 67 000 ± 5000. Chicken and pig mitochondrial malate dehydrogenases, for which mol. wts. of 67 000 and 70 000, respectively, have been reported using ultracentrifugal techniques[20,23], had an elution volume identical to that of the tuna malate dehydrogenases.

Fluorescence spectrum

The fluorescence emission spectra of pig, horse, and chicken mitochondrial malate dehydrogenases all show fluorescence maxima at 307 mμ (refs. 20, 23). Such spectra are typical of proteins lacking tryptophan[24], and the above malate dehydrogenases have also been shown by other criteria to be devoid of this amino acid[20,23]. The fluorescence emission spectrum of the crystalline tuna mitochondrial, shown in Fig. 3, has a maximum at 307 mμ, which indicates that this enzyme also lacks

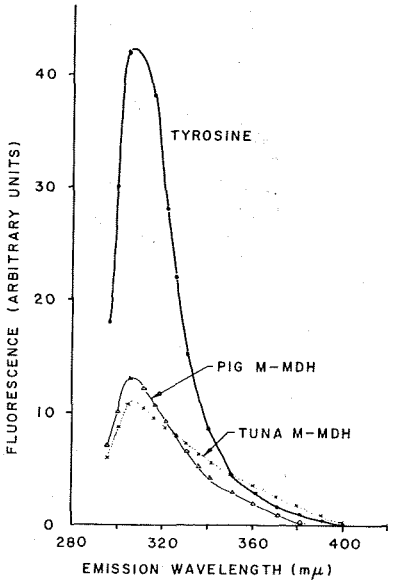


Fig. 3. Fluorescence emission spectra of solutions of tyrosine ($A_{280\ m\mu}$, 0.02), tuna mitochondrial malate dehydrogenase (M-MDH) ($A_{280\ m\mu}$, 0.1), and pig mitochondrial malate dehydrogenase ($A_{280\ m\mu}$, 0.12). Excitation was at 280 mμ.

tryptophan. This is supported by the finding that when peptide maps of tuna mitochondrial malate dehydrogenase were treated with Ehrlich's stain, which is specific for tryptophan[11], no spots gave a positive reaction for this amino acid.

Amino acid composition

The amino acid composition of tuna mitochondrial malate dehydrogenase is shown in Table I, together with the compositions of the chicken supernatant and mitochondrial malate dehydrogenases for comparison. The tuna and chicken mito-

TABLE I

AMINO ACID COMPOSITIONS OF MALATE DEHYDROGENASES

All values are reported as residues per mole (mol. wt. 70 000).

	Tuna mitochondrial malate dehydrogenase	*Chicken mitochondrial malate dehydrogenase**	*Chicken supernatant malate dehydrogenase**
Lys	41 ± 2.6	49	57
His	13 ± 1.9	11	11
Arg	18 ± 1.2	20	20
Asp	60 ± 2.1	52	68
Thr	41 ± 2.0	44	30
Ser	41 ± 3.1	42	35
Glu	50 ± 2.2	60	60
Pro	42 ± 1.8	41	29
Gly	58 ± 1.4	61	59
Ala	72 ± 2.4	66	60
Cys	14**	14	8
Val	65 ± 3.5	48	53
Met	11 ± 1.5	13	14
Ile	33 ± 1.6	40	42
Leu	59 ± 2.1	57	59
Tyr	8 ± 1.2	9	16
Phe	21 ± 0.9	30	24
Trp	0***	0	12

* Data of KITTO AND KAPLAN[23].
** Determined as cysteic acid following performic acid oxidation.
*** From fluorescence spectrum and from staining of peptide maps (see text).

chondrial malate dehydrogenases have very similar amino acid compositions, although the tuna enzyme contains significantly more valine than does the chicken enzyme. The amino acid compositions of the tuna mitochondrial malate dehydrogenase and chicken supernatant malate dehydrogenase show much less similarity.

Peptide maps

The crystalline tuna mitochondrial malate dehydrogenase was subjected to

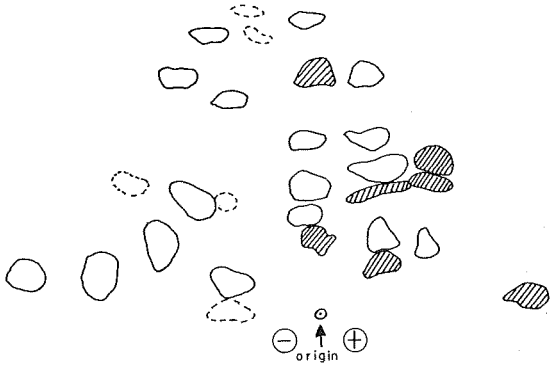


Fig. 4. Peptide map of a tryptic digest of tuna mitochondrial malate dehydrogenase. The sulfhydryl-containing peptides labeled with iodo[^{14}C]acetate are cross-hatched.

tryptic digestion and a peptide map of such a digest is shown in Fig. 4. The peptide maps of this mitochondrial malate dehydrogenase typically showed between 28 and 32 ninhydrin positive spots, about half the number expected on the basis of the total lysine and arginine content. This suggests that the tuna enzyme may be composed of two subunits.

The sulfhydryl groups of the tuna mitochondrial malate dehydrogenase were labeled by incubating the enzyme under nitrogen for several hours with iodo[^{14}C]-acetate, in 8 M urea. The labeled mixture was digested with trypsin, and peptide maps were prepared. The radioactive spots were located by autoradiography of the peptide maps. The location of the sulfhydryl-containing peptides is indicated in the peptide map in Fig. 4. A detailed analysis of the sulfhydryl-containing peptides of the tuna mitochondrial malate dehydrogenase and of malate dehydrogenases from other species will be presented elsewhere.

Extinction coefficient

The extinction coefficient at 280 mμ for the crystalline tuna mitochondrial malate dehydrogenase was determined by measuring the absorbance at 280 mμ in 0.1 M phosphate buffer (pH 7.5) of a carefully dialyzed and clarified solution of the enzyme, and then assaying the nitrogen content of aliquots of the same solution by the Kjeldahl method. By using a value of 16% nitrogen for the enzyme an $E^{1\ mg/ml}_{1\ cm,\ 280\ m\mu}$ of 3.1 was calculated for tuna mitochondrial malate dehydrogenase. The low extinction coefficient reflects the lack of tryptophan in the enzyme and the value obtained is in good agreement with those determined for the mitochondrial malate dehydrogenases of pig[21], horse[21], and chicken[23].

Immunological properties

Rabbit antisera prepared against the crystalline tuna mitochondrial malate dehydrogenase reacted strongly with this enzyme as judged by double diffusion in agar or by microcomplement fixation. No cross-reaction could be observed between this antiserum and tuna supernatant malate dehydrogenase (Fig. 5). The anti-tuna

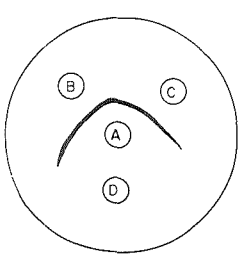


Fig. 5. A tracing of a double diffusion in agar using an antiserum against tuna mitochondrial malate dehydrogenase. The antibody well (A) contained undiluted antiserum (Ra542B-4). The other wells contained solutions of: (B), crystalline tuna mitochondrial malate dehydrogenase; (C), crude tuna heart extract; (D), tuna supernatant malate dehydrogenase.

mitochondrial malate dehydrogenase was also found to inhibit the enzymatic activity of the mitochondrial enzyme, but was without effect on the tuna supernatant malate dehydrogenase.

It was not possible to quantitatively measure the cross-reactions of other fish malate dehydrogenases with the anti-tuna mitochondrial malate dehydrogenase serum using the microcomplement fixation procedure, because many of the fish tissue extracts proved to be strongly anticomplementary. A qualitative measure of such cross-reactions was obtained using double diffusion in agar[14].

The antibody to the tuna mitochondrial malate dehydrogenase cross-reacted strongly with the mitochondrial malate dehydrogenases of species closely related the tuna (*e.g.* mackerel, scup, bass), less strongly with more distantly related fish (*e.g.* herring, trout and salmon) and only very weak cross-reactions were observed with malate dehydrogenases of cartilaginous fish (sharks and dogfish).

Catalytic properties

In order to make a valid comparison of the specific activity of the crystalline tuna mitochondrial malate dehydrogenase with the specific activities of previously

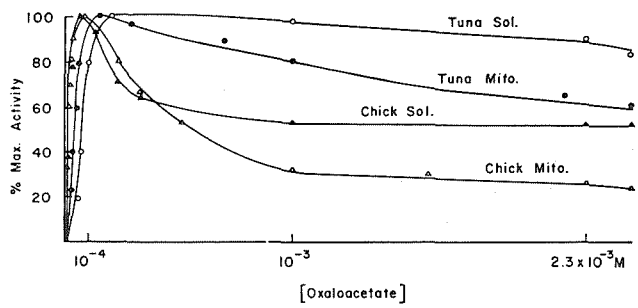


Fig. 6. The effect of oxaloacetate concentration on the activities of tuna supernatant and mitochondrial malate dehydrogenases, and chicken supernatant and mitochondrial malate dehydrogenases.

isolated mitochondrial malate dehydrogenases, assays were performed using the conditions of THORNE AND KAPLAN[20]. Under such conditions the specific activity of tuna mitochondrial malate dehydrogenase was determined to be 730 units per $A_{280\ m\mu}$, which is comparable to the values reported by the above authors for pig and horse

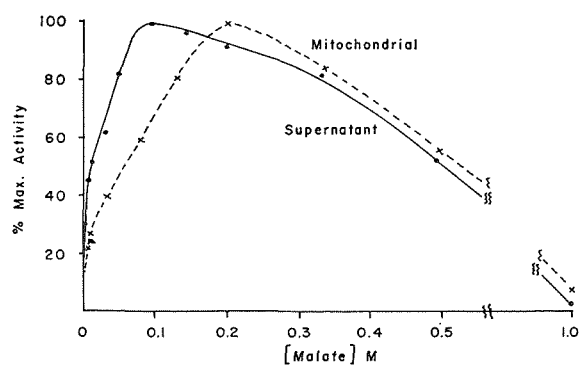


Fig. 7. The effect of malate concentration on the enzymatic activity of tuna supernatant and mitochondrial malate dehydrogenases.

TABLE II

ANALOG RATIOS OF TUNA MALATE DEHYDROGENASES

The analog ratios represent the relative rates of reduction of coenzyme, or coenzyme analogs, at L-malate concentrations of $6 \cdot 10^{-3}$ M (L) or $1 \cdot 10^{-1}$ M (H).

Ratios		*Supernatant*	*Mitochondrial*
$\frac{\text{DPN}}{\text{DPN}}$	(L/H)	0.47	0.28
$\frac{\text{APDPN}}{\text{DPN}}$	(L/L)	2.6	9.2
$\frac{\text{TNDPN}}{\text{DeDPN}}$	(L/L)	2.0	0.5
$\frac{\text{DPN}}{\text{DeDPN}}$	(L/L)	10.0	2.7

mitochondrial malate dehydrogenases. It is also similar to the specific activity of a chicken mitochondrial malate dehydrogenase previously isolated in this laboratory[23].

As is the case with a number of other malate dehydrogenases[4,25,26], the tuna mitochondrial malate dehydrogenase is more susceptible to inhibition by high concentrations of oxaloacetate than is the tuna supernatant malate dehydrogenase. However, this inhibition is observed at markedly higher levels than those required to inhibit the chicken mitochondrial malate dehydrogenase. This is illustrated in Fig. 6. The substrate inhibition characteristics of the tuna malate dehydrogenases

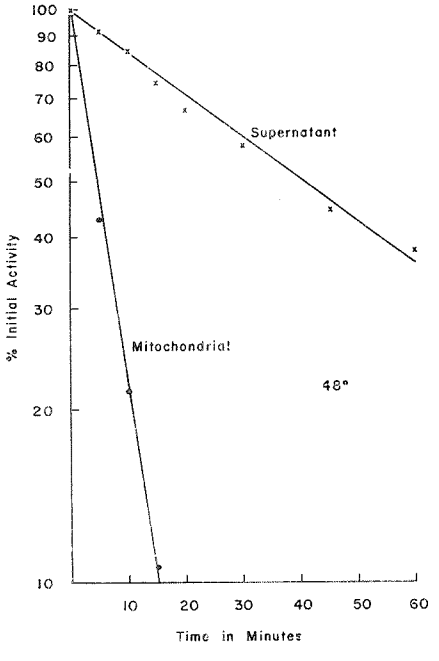


Fig. 8. Thermal inactivation of tuna supernatant and mitochondrial malate dehydrogenases at 48°.

with varying levels of malate is shown in Fig. 7. Although the two tuna enzymes require different concentrations of malate for optimal activity, both show similar degrees of inhibition at high malate concentrations. As was the case with oxaloacetate, very high malate concentrations were required to produce significant inhibition of the tuna malate dehydrogenases.

Pyridine coenzyme analogs have been used in this laboratory for a number of years to show the heterogeneity of enzymes catalyzing the same chemical reaction[27]. The results, presented in Table II, for the tuna supernatant and mitochondrial malate dehydrogenases, show clearly that these two enzymes can also be differentiated on the basis of their reactivity with coenzyme analogs. The same analog ratios were obtained with crystalline tuna mitochondrial malate dehydrogenase and a sample of this malate dehydrogenase purified to the same degree as the supernatant malate dehydrogenase, indicating that the differences observed between the two enzymes were not due to differences in purity.

Results of heat inactivation experiments with the tuna malate dehydrogenases are presented in Fig. 8. The tuna mitochondrial malate dehydrogenase is more thermolabile than is the supernatant malate dehydrogenase, but the difference in heat stability of the two malate dehydrogenases is much less marked than with the heart and muscle types of lactate dehydrogenase[10]. The temperature required for inactivation of tuna mitochondrial malate dehydrogenase (48°) is slightly less than that required to inactivate chicken mitochondrial malate dehydrogenase (55°) (ref. 23).

Hybridization studies

Apart from our interest in studying the physical, catalytic, and immunological properties of tuna malate dehydrogenases from a comparative standpoint, our decision to purify these enzymes was prompted also by their unusual electrophoretic properties. As shown in Fig. 1, the tuna mitochondrial malate dehydrogenase migrates a considerable distance towards the anode when subjected to starch-gel electrophoresis at pH 7.0. In this respect it differs from the mitochondrial malate dehydrogenase of chicken, which remains near the point of application on the gel, and from pig mitochondrial malate dehydrogenase which migrates slightly towards the cathode at this pH. The electrophoretic properties of the tuna mitochondrial malate dehydrogenase enabled us to perform a number of experiments designed to produce hybrid malate dehydrogenases. The details of such experiments have been reported fully elsewhere[28,29], but for the sake of completeness the results of a chicken

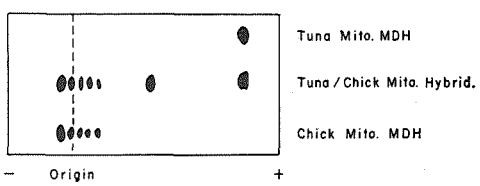


Fig. 9. Tracing of a starch-gel electrophoresis showing the hybridization of tuna and chicken mitochondrial malate dehydrogenases (MDH). The enzymes were dissociated in 7.6 M guanidine·HCl containing 0.1 M sodium citrate, 0.1 M β-mercaptoethanol (pH 7.0). Reactivation was in 0.1 M sodium citrate, 0.1 M β-mercaptoethanol (pH 7.0). The chicken and tuna enzymes were reactivated separately as controls and a 1:1 mixture of the two enzymes was reactivated in the hybridization experiment.

mitochondrial malate dehydrogenase–tuna mitochondrial malate dehydrogenase hybridization are illustrated in Fig. 9. The catalytic and immunological properties of the hybrid were found to be intermediate between those of the parent enzymes[29].

DISCUSSION

While there have been reports on the purification and properties of malate dehydrogenases from a wide variety of sources, including human[30], pig[21], beef[22,31,32], horse[21], rat[4,33], pigeon[21], chicken[23], *Neurospora crassa*[34], and *Bacillus subtilis*[35], among others, we believe that the present investigation is the first to examine in detail the properties of the malate dehydrogenases of a fish. As such, it is of interest to compare the properties of the tuna enzymes with those derived from other sources.

In a number of respects the tuna malate dehydrogenases resemble those of other vertebrate species. The tuna mitochondrial malate dehydrogenase has an amino acid composition which closely resembles those of the pig, horse, and chicken mitochondrial enzymes, the most striking feature being the lack of tryptophan. As in the case of the chicken malate dehydrogenases[23] the mitochondrial tuna malate dehydrogenase is the most heat-labile form. The specific activity of the crystalline tuna mitochondrial malate dehydrogenase is similar to that reported for other vertebrate malate dehydrogenases. The distinction of the supernatant and mitochondrial malate dehydrogenases of tuna, on the basis of their analog ratios and immunological properties, is also reminiscent of the findings with the enzymes of their vertebrate relatives.

The tuna enzymes do, however, differ from their higher vertebrate counterparts in that, with the fish enzymes, markedly higher concentrations of oxaloacetate and malate are required to produce significant substrate inhibition, under similar assay conditions.

Another point of interest is that the tuna supernatant and mitochondrial malate dehydrogenases, when compared with, for example, the analogous enzymes of pig and chicken, show an opposite electrophoretic migration of the two enzyme types. Such switches in the electrophoretic mobility of enzyme forms catalyzing the same reaction dramatically illustrate the impracticability of naming multiple enzyme forms solely on the basis of their electrophoretic behavior.

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